Ex Vivo and In Vivo Regulation of Lipocalin-2, a Novel Adipokine, by Insulin

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OBJECTIVE — Lipocalin-2, a novel adipokine, has been shown to be elevated in obese, insulin-resistant, and diabetic subjects. We therefore sought to study the ex vivo and in vivo effects of insulin on lipocalin-2 levels in humans.

RESEARCH DESIGN AND METHODS — We investigated the in vivo effects of insulin (hyperinsulinemia) on circulating lipocalin-2 levels by enzyme-linked immunosorbent assay via a prolonged insulin-glucose infusion. The ex vivo effect of insulin on adipose tissue lipocalin-2 protein production and secretion into conditioned media was assessed by Western blotting and enzyme-linked immunosorbent assay, respectively.

RESULTS — Hyperinsulinemic induction in human subjects significantly increased circulating lipocalin-2 levels ($P < 0.01$). Also, in omental adipose tissue explants, insulin caused a significant dose-dependent increase in lipocalin-2 protein production and secretion into conditioned media ($P < 0.05$, $P < 0.01$, respectively); these effects were negated by both phosphatidylinositol 3-kinase and mitogen-activated protein kinase kinase inhibitors.

CONCLUSIONS — Lipocalin-2 is upregulated by insulin via phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways.

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Obesity and the metabolic syndrome are associated with serious cardiometabolic sequelae including insulin resistance, hyperinsulinemia, diabetes, dyslipidemia, and cardiovascular disease (1). The metabolic syndrome is associated with visceral obesity. Adipose tissue produces cytokines termed "adipokines" that are implicated in the pathogenesis of the metabolic syndrome (2).

Recently, Yan et al. (3) established lipocalin-2 as a novel adipokine, highly expressed by adipose tissue in murine models of obesity. Also, they demonstrated that lipocalin-2 levels are increased by dexamethasone and tumor necrosis factor-α and are reduced by rosiglitazone in murine adipocytes (3). More recently, Wang et al. (4) reported elevated levels of lipocalin-2 in obesity and diabetes.

We therefore studied the effects of acute and chronic hyperinsulinemia on circulating lipocalin-2 levels via a prolonged insulin-glucose infusion in humans. We also assessed the effects of insulin on lipocalin-2 protein production and secretion into conditioned media from human visceral adipose tissue explants.

RESEARCH DESIGN AND METHODS

— We measured circulating lipocalin-2 in six healthy subjects (mean ± SD age 26.5 ± 8 years and BMI 23.2 ± 2.5 kg/m^2). To account for the possible diurnal variation in lipocalin-2 levels, we obtained a daily control curve by measuring fasting lipocalin-2 levels at 30-min intervals from 0800 to 1000 h. Subsequently, lipocalin-2 levels were measured at 2-h intervals until 2400 h and then at 0400 h as well as at 30-min intervals from 0800 to 1000 h on day 2. On the following day, the same subjects were subjected to a prolonged insulin-glucose infusion for 26 h beginning at 0800 h. Insulin (human Actrapid) was administered intravenously as a priming dose of 0.04 units/kg followed by continuous infusion of 0.5 mU · kg^{-1} · min^{-1}. By choosing this rate of insulin infusion, we expected to achieve hyperinsulinemia with an approximate four- to sixfold elevation of basal insulinemia (5). Fasting blood samples were drawn at 30-min intervals between 0800 and 1000 h on day 1 and day 2 of the prolonged insulin-glucose infusion (the first and the last 2 h of the infusion). Intermediate blood samples were taken at 2-h intervals until 2400 h and then at 0400 h on day 2. Glucose levels were maintained between 4.0 and 6.0 mmol/l.

For adipose tissue explant studies, after an overnight fast, adipose tissue was obtained (0800–1000 h) from six surgical patients (age 27.5 ± 7 years and BMI 23.8 ± 2.8 kg/m^2). Samples were placed into sterile containers containing Medium 199 (Sigma-Aldrich, Gillingham, U.K.) for primary adipose tissue culture.

Exclusion criteria for all subjects included known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes, hypertension (blood pressure >140/90 mmHg), and renal impairment (serum creatinine >120 μmol/l). None of the subjects was on any medications for ≥6 months before the study, including glucocorticoids, antidiabetes drugs, antiobesity drugs, and lipid-lowering agents or antihypertensive medication. The local research ethics committee approved the study, and all patients involved gave their informed consent, in accordance with the guidelines in the Declaration of Helsinki, 2000.

Assays

Serum insulin levels were measured by radioimmunoassay (Pharmacia, Milton Keynes, U.K.). Lipocalin-2 in serum and conditioned media from human omental

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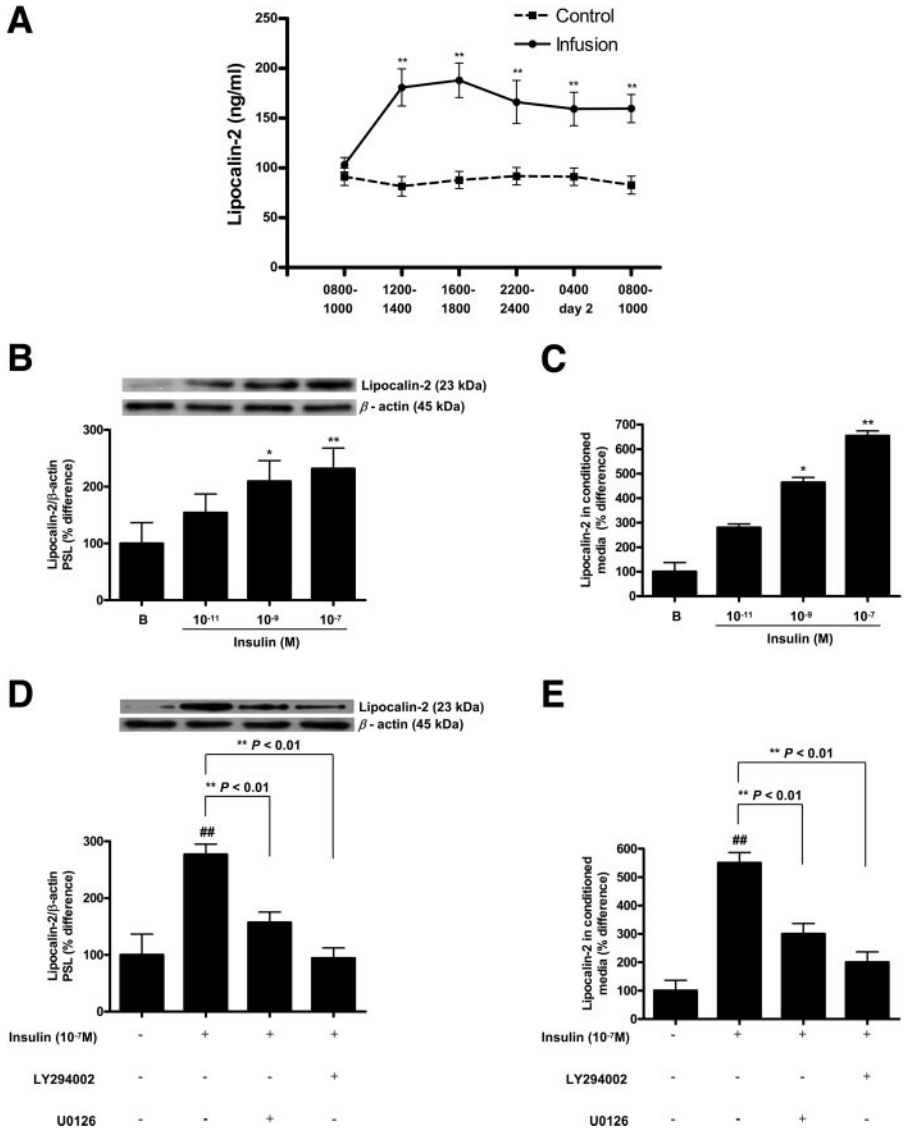

Figure 1—A: *Mean concentrations of lipocalin-2 in ng/ml in all subjects before and after insulin infusion. Data are means ± SD. Group comparison by Student's* t *test.* **P $<$ *0.01.* B: *Dose-dependent effects of insulin (10^{-11}, 10^{-9}, and 10^{-7} mol/l) on lipocalin-2 protein production in human omental adipose tissue explants at 24 h were assessed by Western blotting. Western blot analysis of protein extracts from omental adipose tissue demonstrates that the antibody against lipocalin-2 and the antibody against β-actin recognized bands with apparent molecular weights of 23 and 45 kDa, respectively. Densitometric analysis of lipocalin-2 immune complexes normalized to β-actin revealed that protein levels of lipocalin-2 were significantly increased by insulin (10^{-9} and 10^{-7} mol/l) in human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison was by Friedman's ANOVA and post hoc Dunn's test.* *P $<$ *0.05;* **P $<$ *0.001. PSL, phosphostimulated light units.* C: *Dose-dependent effects of insulin (10^{-11}, 10^{-9}, and 10^{-7} mol/l) on lipocalin-2 secretion into conditioned media from human omental adipose tissue explants at 24 h were measured by ELISA. Lipocalin-2 secretion was significantly increased by (10^{-9} and 10^{-7} mol/l) human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison was by Friedman's ANOVA and post hoc Dunn's test.* *P $<$ *0.05;* **P $<$ *0.001.* D: *Effect of PI3K (LY294002) and MEK (U0126) inhibitors on insulin-induced lipocalin-2 protein production in human omental adipose tissue explants at 24 h was assessed by Western blotting and compared with insulin (10^{-7} mol/l) without inhibitors. Western blot analysis of protein extracts from omental adipose tissue demonstrates that the antibody against lipocalin-2 and the antibody against β-actin recognized bands with apparent molecular weights of 23 and 45 kDa, respectively. Densitometric analysis of lipocalin-2 immune complexes normalized to β-actin revealed that insulin-induced lipocalin-2 protein production was significantly decreased by LY294002 and U0126 in human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Differences between groups were assessed using the Mann-Whitney* U *test.* **P $<$ *0.01;* ##P $<$ *0.01.* E: *Effect of PI3K (LY294002) and MEK (U0126) inhibitors on insulin-induced lipocalin-2 levels in conditioned media from human omental adipose tissue explants at 24 h was assessed by ELISA and compared with insulin (10^{-7} mol/l) without inhibitors. Lipocalin-2 secretion into conditioned media was significantly decreased by LY294002 and U0126 from human omental adipose tissue explants. Data are expressed as percent difference of median of basal. Each experiment was carried out with six different samples from six different subjects in three replicates. Differences between groups were assessed using the Mann-Whitney* U *test.* **P $<$ *0.01;* ##P $<$ *0.01.*

adipose tissue explants was measured by ELISA (R&D Systems, Abingdon, U.K.), according to the manufacturer's protocol, with an intra-assay coefficient of variation of <5%.

Primary explant culture

Adipose tissue explants were cultured with or without the addition of insulin (Sigma-Aldrich), mitogen-activated protein kinase kinase (MEK) inhibitor (U0126; Calbiochem, San Diego, CA), or phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002; Calbiochem) as previously described (6).

Western blotting

Protein lysates were prepared and Western blotting was performed as previously described (6). We used monoclonal primary mouse anti-human antibody for lipocalin-2 (Abcam, Cambridge, U.K.) (1:500 dilution) and monoclonal primary rabbit anti-human antibody for β-actin (Cell Signaling Technology, Beverly, MA) (1:1,000 dilution).

Statistics

Data were analyzed by Student's *t* test, Mann-Whitney *U* test, and/or Friedman's ANOVA according to the number of groups compared. $P < 0.05$ was considered significant.

RESULTS — Insulin infusion resulted in elevation of fasting insulinemia from 78.1 ± 12.0 pmol/l to 294.6 ± 31.0 pmol/l. Insulin levels remained elevated until the end of the prolonged insulin-glucose infusion (366.0 ± 37.0 pmol/l). Lipocalin-2 levels remained unaltered throughout the control day from 91.2 ± 21.5 ng/ml between 0800 and 1000 h to 82.8 ± 21.9 ng/ml between 0800 and 1000 h the next day (Fig. 1*A*; $P > 0.05$).

There was a profound effect of insulin on lipocalin-2 levels over 26 h of insulin infusion: from 103.2 ± 17.9 ng/ml between 0800 and 1000 h to 159.7 ± 34.5 ng/ml between 0800 and 1000 h the following day (Fig. 1*A*; $P < 0.01$). The increase in lipocalin-2 levels was relatively acute approaching maximal values at 4 h (180.8 ± 45.7 ng/ml) (Fig. 1*A*; $P < 0.01$) and persisting throughout the entire period of hyperinsulinemia.

Lipocalin-2 protein production and secretion into conditioned media were significantly increased dose dependently by insulin from human omental adipose tissue explants; these effects were negated by both PI3K inhibitor (LY294002; 50 μmol/l) and MEK inhibitor (U0126; 10 μmol/l) (Fig. 1*B–E*; $*P < 0.05$, $**P < 0.01$, $\#\#P < 0.01$). Treatment of adipose tissue explants with either LY294002 or U0126 alone did not affect lipocalin-2 levels (data not shown).

CONCLUSIONS — Our findings highlight the involvement of both PI3K and mitogen-activated protein kinase signaling pathways in insulin-induced lipocalin-2 production and may explain the increased lipocalin-2 levels in hyperinsulinemic subjects (4). Our observations may have clinical/therapeutic applications, given that lipocalin-2 promotes insulin resistance in adipocytes and hepatocytes (3).

It should be emphasized that our study utilized relatively small numbers of subjects because of the challenge imposed by the prolonged insulin-glucose infusion study. Additionally, we studied a relatively short-term effect of hyperinsulinemia (24 h) in healthy subjects because type 2 diabetes and insulin resistance syndromes are more chronic states of hyperinsulinemia. Nevertheless, our observations are highly consistent and significant and raise interesting questions on the mechanisms regulating lipocalin-2 production.

In conclusion, we show for the first time the potent and robust regulation of lipocalin-2 by insulin ex vivo and in vivo. Our findings provide novel insights into lipocalin-2 physiology, which may be pertinent to hyperinsulinemic states such as obesity and diabetes.

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No potential conflicts of interest relevant to this article wre reported.

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References

1. Eckel RH, Grundy SM, Zimmet PZ: The metabolic syndrome. *Lancet* 365:1415–1428, 2005
2. Kershaw EE, Flier JS: Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 89:2548–2556, 2004
3. Yan QW, Yang Q, Mody N, Graham TE, Hsu CH, Xu Z, Houstis NE, Kahn BB, Rosen ED: The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance. *Diabetes* 56:2533–2540, 2007
4. Wang Y, Lam KS, Kraegen EW, Sweeney G, Zhang J, Tso AW, Chow WS, Wat NM, Xu JY, Hoo RL, Xu A: Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. *Clin Chem* 53:34–41, 2007
5. Lewandowski K, Randeva HS, O'Callaghan CJ, Horn R, Medley GF, Hillhouse EW, Brabant G, O'Hare P: Effects of insulin and glucocorticoids on the leptin system are mediated through free leptin. *Clin Endocrinol (Oxf)* 54:533–539, 2001
6. Tan BK, Chen J, Lehnert H, Kennedy R, Randeva HS: Raised serum, adipocyte and adipose tissue Retinol-Binding Protein 4 (RBP4) in overweight women with polycystic ovary syndrome: effects of gonadal and adrenal steroids. *J Clin Endocrinol Metab* 92:2764–2772, 2007